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14. ABSTRACT

A significant achievement in the current reporting period is our ability to immunostain both the 12-LOX protein and β4 integrin in paraffin-embedded prostate tumor tissues circumventing the problems described in the previous report. With the new protocol we have stained about 20 cases so far and the remaining cases are in progress. We have generated several stable transfectants of PC-3 cells expressing mutant forms of the β4 integrin and studied their interaction with 12-LOX. During this study, we have identified that the peptide spanning between amino acids 1126 and 1315 of the cytoplasmic tail of the β4 integrin shows strong interaction with 12-LOX. An important observation is that the full-length cytoplasmic tail of β4 integrin, when expressed ectopically, disrupts the interaction with 12-LOX with β4 integrin in a dominant negative manner. This interaction also resulted in a decrease in the biosynthesis of the enzymatic product, 12-HETE, as well as reduction in the tumor growth rate from subcutaneously injected PC-3 cells in athymic nu/nu mice.

15. SUBJECT TERMS
Prostate cancer, hemidesmosome, 12-lipoxygenase, HGF, SF

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INTRODUCTION

During the progression of human PCa hemidesmosomes, adhesion structures that anchor epithelial cells to basement membrane and function as a tumor suppressor, are lost (1, 2). We found that 12-lipoxygenase directly interacts with β4 integrin, an integral part of hemidesmosomes (3, 4). We hypothesized that an increase in 12-LOX activity can cause the disassembly of hemidesmosomes, mobilization of α6β4 integrin from hemidesmosomes to other parts of the cell membrane, and stimulate tumor invasive growth. We proposed to conduct a correlation study to evaluate the relationship between 12-LOX expression and dispersion of β4 integrin in clinical tumor specimens. Our proposal further aims to study whether 12(S)-HETE, the enzymatic product of 12-LOX, can disrupt hemidesmosomes and whether 12-LOX inhibitors promote the formation of hemidesmosomes. Then we will study the underlying signaling pathway, especially PKCα, initiated by 12(S)-HETE, in the disassembly of hemidesmosomes. Next we will overexpress β4 integrin and study the role of the interaction between 12-LOX and β4 integrin in the adhesion, proliferation, migration, and survival, in response to HGF/SF. Finally we will xenograft these transfected cells into mice, to evaluate whether any phenotypic changes of tumor cells in vitro can be recapitulated in vivo. The work will significantly advance our understanding about the complex process of prostate cancer progression as well as the possible role played by dietary fat in the progression of prostate cancer.

BODY OF PROGRESS REPORT

Task 1.

We have attempted several procedures for immunostaining for 12-LOX. In the previous report, the procedure only worked in frozen human prostate tumor tissue. We now worked out the conditions for immunohistochemical analysis of 12-LOX at the protein level in paraffin-embedded human prostate tumor tissues. As shown in the figure 1, 12-LOX immunoreactivity correlated with tumor grade. Neoplastic glands are weakly, moderately or strongly positive for 12-LOX in hyperplatic glands (**Figure 1A**), atropic glands (**Figure 1 B**), and in tumor Glenson score 4-6 (**Figure 1 C,D**).

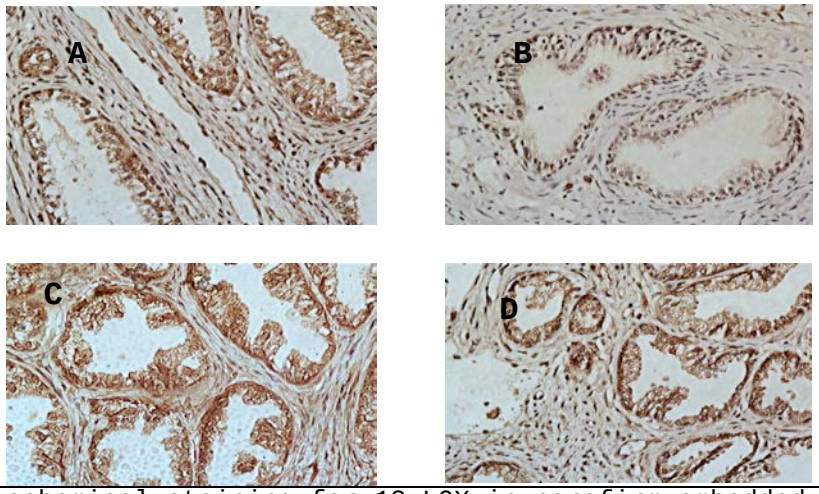


Figure 1. Immunohistochemical staining for 12-LOX in parafirm-embedded prostate tumor tissues. Sections of specimens were probed with a12-LOX polyclonal antibody (Oxford Biomedical Research Inc, Oxford, MI). Positive immunoreactivity is indicated by staining with brownish color. A, hyperplasia gland; B, atropic; C/D, tumor with Glenson score 4-6.

We have also attempted several protocols of immunostaining for β4 integrin in paraffin-embedded material. As shown in figure 2, positive staining was found in tumors and correlated with tumor grade.

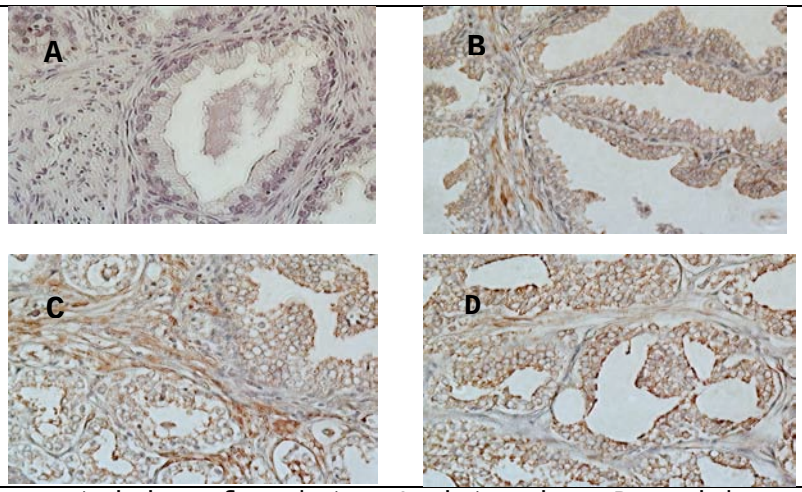


Figure 2. Immunostaining for beta 4 integrin. Brownish color indicates positive staining. Note the pattern of beta 4 integrin positivity. A, normal epithelium; B, tumor with Glenson score 4; C, tumor with Glenson score 6; D, tumor with Glenson score 8.

We have procured 100 cases of prostate tumor specimens. We have analyzed 83 patient samples following the double staining technique. The results are presented in **Table 1**.

Table 1: 12-LOX and b4 Expression in Human Prostate Cancer Compared to Gleason Score

Gleason Score	n	β4 Median (range)	12-LOX Median (range)
6	42	30 (5-80)	15 (5-70)
≥7	41	70 (5-100)	30 (5-80)
p value		0.0082	0.0109

Mann-Whitney test used for statistical analysis. $p \leq 0.05$ was considered significant.

Task 2. Study the effects of 12-LOX inhibitors and 12(S)-HETE on hemidesmosome in prostate epithelium. Months 1-18:

We studied the effect of inhibitors of 12-LOX as well as 12(S)-HETE on the hemidesmosome-like structures in cultured cells. As shown in Figures 3 and 4, 12(S)-HETE (300 nM) reduced hemidesmosome like structures both in A431 cells and in an immortalized human prostate cancer cell line, CPTX1532. In contrast, baicalein, a 12-LOX inhibitor, promoted the formation of hemidesmosome-like structure (Figure 4). Interestingly, a linoleic acid metabolite from 15-LOX, 13(S)-HODE, which is known antagonist of 12(S)-HETE, promoted the formation of hemidesmosomes (Figure 3).

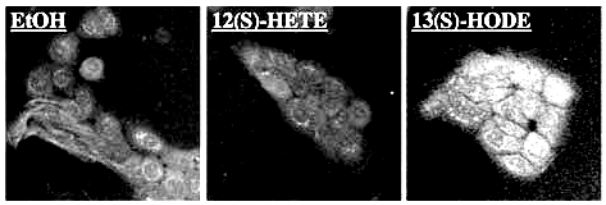
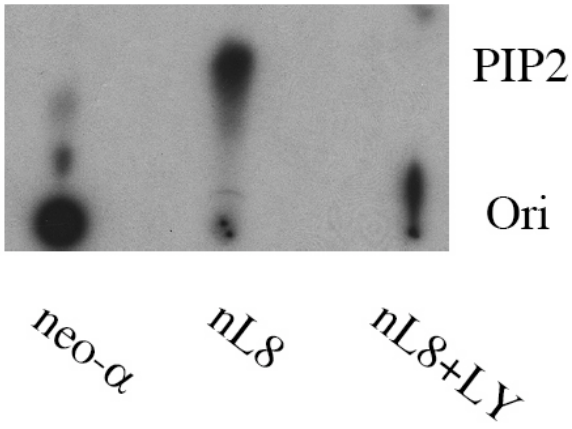


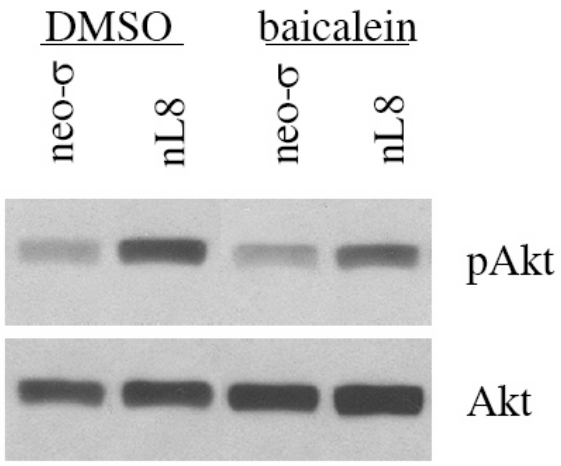
Figure 3: 12(S)-HETE reduced the focal staining of β4 integrin (middle) while 13(S)-HODE promoted the focal staining (right) in A431 cells. A431 cells were plated on matrigel and treated with 300 nM of 12(S)-HETE or 13(S)-HODE for 30 min, fixed with methanol/acetone, and then processed for immunostaining for β4 integrin. Note the marked increase in focal staining for β4 integrin in 13(S)-HODE treated group (right) vs. the control (EtOH, left). Also note the marked reduction in focal staining for β4 in 12(S)-HETE treated group (middle) when compared to the control (left).

Figure 4: 12(S)-HETE reduced (right), while 12-LOX inhibitor baicalein promotes (middle), the polarized staining of β4 integrin in CPTX1532, a human prostate epithelial cell line immortalized with the E6 and E7 transforming proteins of human papilloma virus serotype 16 (Bright et al., 1997). Cells were treated with baicailein or 12(S)-HETE for 30 min, fixed with methanol/acetone, and then processed for immunostaining for β4 integrin. Note the focal, or polarized staining pattern in baicalein treated group, and less in control group. Cells treated with 12(S)-HETE represented much less focal staining.

Task 3. Study the signal transduction pathways that underlie the disassembly of hemidesmosomes by 12(S)-HETE or an increase in 12-LOX activity, Months 12 -24:



Increased PI3 kinase activity in PC-3 cells by 12-LOX overexpression. PI3 kinase activity in p85 immunoprecipitates was measured by incubating with substrate and γ-32P-ATP. Phospholipids were separated by TLC and autoradiographed. Shown here is a representative of three independent experiments. Note the increased PIP2 production in nL8 when compared to neo and its sensitivity to a PI3 kinase inhibitor, LY294002 (LY). Ori, origin.



Increased Akt phosphorylation in 12-LOX transfected PC-3 cells and its attenuation by baicalein. Neo-α and nL8 cells were treated with DMSO or 15 μM of baicalein for 16 h. Cell lysates were harvested and the phosphorylation of Akt was analyzed by using a polyclonal antibody against pSer473 Akt (Top panel). Bottom panel, the membrane was stripped and reprobed for total Akt (phosphorylated and nonphosphorylated).

Fig 5. Activation of the PI3K Akt signaling pathway in 12-LOX overexpressing PC-3 cells. **A**. **B**.

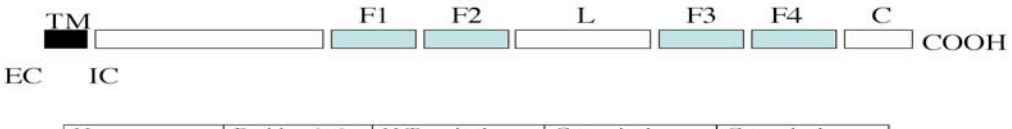
In Vitro PI3 - Kinase Assay - In vitro phosphorylation of phosphatidylinositols was carried out with PY20 antibody (Transduction laboratory, KY) for the detection of immunoprecipitated PI 3-kinase essentially as previously described (23). Prostate cancer cells were harvested with PBS containing 1 % Nonidet P-40, 1 mM dithiothreitol, 200 μM sodium vanadate, 100 μM 4-(2- aminoethyl) benzenesulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin (lysis buffer). Soluble cell lysates were collected after microcentrifugation at 18,000 g for

5 min. The protein concentration of cell lysates was adjusted to 0.8-1 µg/µl with lysis buffer and PY20 mAb (5 µg) was added to 500 µl of the cell lysate. The mixture was incubated with gentle rocking at 4 °C overnight and then 10 mg of protein A - Sepharose (Amersham Pharmacia Biotech) were added and the incubation was continued for another 2 h. The immunoprecipitates were washed (3 x) with the aforementioned lysis buffer, twice with 0.1 M Tris/HCl, pH 7.5, containing 0.5 M LiCl, and 10 µM sodium vanadate, and twice with 10 mM Tris/HCl, pH 7.5, containing 100 mM NaCl, 10 µM sodium vanadate, and 1 mM EDTA. Then 50 µl kinase buffer [10 mM HEPES (pH7.0), 0.1 mM EGTA, 25 mM MgCl2, 100 µM ATP, 10 µCi of [32P]ATP, 10 µg/sample PtdIns] was added to each sample, and incubated at room temperature for 10 min. The assay was terminated by adding 60 µl of 2 µM HCl and 160 µl of chloroform/methanol (1:1) / sample, and followed by vortexing and centrifugation. The chloroform phase which contained the inositides was removed and applied to Silica gel 60 thin layer chromatography (TLC) sheets (Merck). TLC was developed with chloroform : methanol : 24% ammonium hydroxide : H2O (90 : 90 : 9 : 19). After drying, spots were located by autoradiography and compared with standards.

Task 4. Overexpress b4 integrin in PC-3 cells, in the presence or absence of 12-LOX expression, and evaluate the capacity of transfected cells to form hemidesmosomes and whether an increase in surface expression of a6b4 alters cell proliferation, adhesion, migration, and survival, in response to HGF/SF, Months 18 - 30:

Generation of PC-3 cells expressing various β4 mutants: We constructed a panel of bacterial expression plasmids and mammalian expression constructs that express various b4 mutants of the cytoplasmic tail as shown in Figure 6. Using pGR30~47, we have generated a panel of stable transfectants that express various b4 mutants as GFP fusion proteins through fluorescence activated cell sorting (FACS) and G418 selection for the transfectants.

The expression of β4 mutants as GFP-fusion proteins is shown in figure 7. Co-immunoprecipitation was performed to determine the interaction of 12-LOX with various β4 mutants. As shown in the figure 8, 12-LOX is able to bind to the fusion proteins expressed by pGR40, pGR45, pGR46, and pGR47, but not to the fusion protein with only the linker region (pGR38). 12-LOX weakly binds to the fusion protein encoded by pGR39. Collectively, the data suggest a strong binding site(s) for 12-LOX located between 1126–1315 of β4 cytoplasmic tail.



Name	Position (aa)	N-Terminal HIS-tag, T7 promoter	C-terminal myc-tag, CMV promoter	C-terminal EGFP, CMV promoter
$ß_4$-$F_{1,2}$	1126-1315	pGR1	pGR16	-
$ß_4$-$F_{1,2}L$	1126-1457	pGR36	-	pGR45
$ß_4$-$F_{1,2}L(F_3)$	1126-1485	pGR5	-	pGR40
$ß_4$-F_1	1126-1218	pJP1	-	-
$ß_4$-F_2	1219-1314	pJP2	-	-
$ß_4$-L	1316-1457	pGR2	pGR17	pGR38
$ß_4$-F_2L	1219-1457	pGR4	pGR18	-
$ß_4$-F_3	1457-1546	pJP3	-	-
$ß_4$-F_4	1570-1662	pJP4	-	-
$ß_4$-$F_{3,4}$	1457-1662	pJP5	-	-
$ß_4$-$F_{(3),4}C$	1486-1752	pGR3	pGR13	pGR39
$ß_4$-$LF_{3,4}C$	1316-1752	pGR37	-	pGR41
$ß_4$-$F_{3,4}C$	1458-1752	pGR44	-	-
$ß_4$-$F_{1,2}LF_{3,4}C$	1126-1752	pGR6	pGR20	pGR30
$ß_4$-TM-F_2	708-1315	pGR42	-	pGR46
$ß_4$-TM-C	708-1752	pGR43	-	pGR47

F... Fibronectin type3-like repeat
L... Linker region between fibronectin pairs
C... C-terminal region (C-terminal of second fibronectin pair)
TM... Transmembrane domain

Figure 6. Schemes of the cytoplasmic tail of β4 integrin and bacterial and mammalian expression constructs. TM, transmembrane; EC, extracellular; IC, intracellular

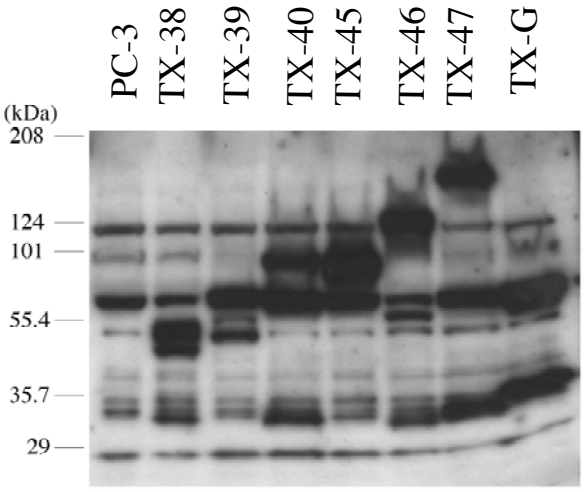


Figure 7. Western blot analysis for the expression of β4 mutants in PC-3 transfectants with an antibody against GFP. Note the expression of GFP fusion proteins of various MWs. The calculated MW for fusion proteins for pGR47 (Tx47) is about 146. For pGR46 (Tx46), 97.4; pGR45 (Tx45), 66.8; pGR40 (Tx40), 70; pGR39 (Tx39), 59.6; and pGR38 (Tx38), 45.7. The MW for pEGFP (TxG) is approximately 30.

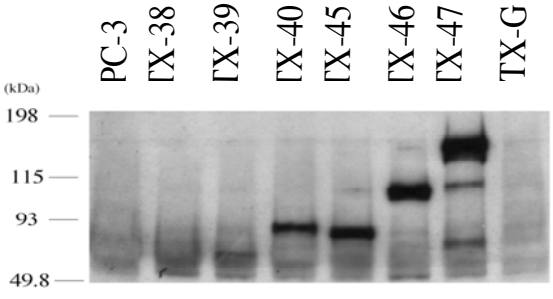


Figure 8. Co-immunoprecipitation of 12-LOX with the fusion proteins expressed by pGR40, pGR45, pGR46, and pGR47, but not to pGR38 and pEGFP (TxG). It is deduced that a binding site or sites are located between 1126 – 1315 region of the β4 cytoplasmic tail.

Cytosolic β4 cytoplasmic tail binds to 12-LOX and blocks the interaction between 12-LOX and full length β4 integrin: As shown above, we have constructed a panel of expression constructs encoding various mutants for the β4 cytoplasmic tail. The construct pGR30 encodes the cytosolic β4 cytoplasmic tail, with TM domain deleted, as a GFP-fusion protein (Figure 6). When ectopically expressed in 12-LOX transfected PC-3 cells, the cytosolic β4 cytoplasmic tail interacts with 12-LOX (Figure 9, the IP: 12-LOX / IB: GFP panel). Interestingly the ectopically expressed cytosolic β4 tail blocks the interaction of 12-LOX with full-length β4 integrin (Figure 9, the IP: 12-LOX / IB: β4 panel). The blockade of the interaction between 12-LOX and full-length β4 integrin is not due to the lack of β4 expression (Figure 9, the IB: β4 panel), but due to the presence of the cytosolic β4 cytoplasmic tail (Figure 9, the IB: GFP panel). The results suggest that the cytosolic β4 mutant, encoded by pGR30, is able to block the interaction between 12-LOX and full-length β4 integrin in a dominant negative manner.

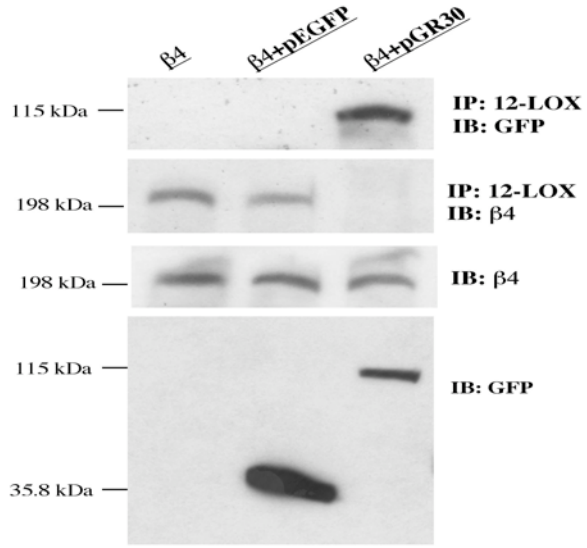


Figure 9: Cytosolic β4 tail, encoded by pGR30, binds to 12-LOX and blocks the interaction between 12-LOX and full length β4 integrin. 12-LOX transfected PC-3 cells (PC3/12LOX) were transiently transfected with an expression construct for full-length β4, in the presence or absence of pGR30 or its vector, pEGFP. Cell lysates were immunoprecipitated with a polyclonal antibody against 12-LOX and immunoblotted for GFP (JL8 mAb, Clontech) or full-length β4 integrin (AB1922, Chemicon International. Target band, around 205 kDa). Total lysates were also analyzed for the expression of full-length β4 integrin and GFP or GFP-fusion protein by immunoblot. Note the co-immunoprecipitation of 12-LOX and the cytosolic β4 cytoplasmic tail and the blockade of the interaction between 12-LOX and full-length β4 integrin by co-transfection with pGR30.

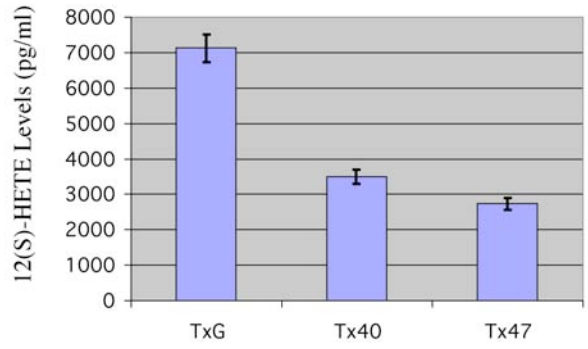
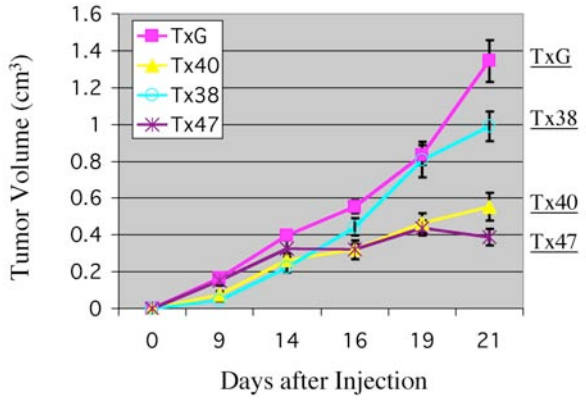


Figure 10. Decrease in 12(S)-HETE biosynthesis in PC-3 cells that express 12-LOX binding β4 mutants. 12(S)-HETE was isolated from cell lystaes and measured by EIA. N = 6 for each type of cells. Bar, SEM.



Figigure 11. Alteration in PC-3 tumor growth as a result of ectopic expression of 12-LOX binding β4 mutants (Tx40 and Tx47), not non-12LOX-bind mutant (Tx38). TxG is the vector control. Each datum point, the average tumor volume from 4 mice. Bar, SEM. Note the correlation of the reduction in tumor growth with reduced 12(S)-HETE biosynthesis shown in figure 7.

Task 5. Evaluate the growth rates of s.c. tumors derived from α6β4 expressing PC-3 cells, in the presence or absence of stable 12-LOX expression, and compare with that of control PC-3 cells, Months 24-36:

We have conducted a preliminary study to determine whether ectopic expression of β4 mutants, especially those that can bind 12-LOX, can effect 12-LOX activity. As shown in Figure 10, there was a reduction in 12(S)-HETE biosynthesis in PC-3 cells expressing two 12-LOX binding β4 mutants (pGR40 and pGR47). We are still in the process of determining whether 12-LOX cellular localization and interaction with the full-length β4 integrin are altered as result of the presence of β4 mutants encoded by pGR40 and pGR47. When injected into athymic nu/nu mice, it was found, as shown in Figure 11, that there were a reduction in tumor growth rate in PC-3 cells expressing 12-LOX binding β4 mutants (Tx40 and Tx47, see figure 11), when compared to those from vector control (TxG) or a β4 fragment that does not interact with 12-LOX (Tx38, see Figure 11).

Key Research Accomplishments:

- Optimized the conditions for immunohistochemical analysis of 12-LOX and β4 at the protein level in paraffin-embedded human prostate tumor tissues. This standardized method had enabled

us to continue with the correlation between 12-LOX expression and distribution of β4 integrin with Gleason score.
- Constructed several β4 cytoplasmic tail mutants and identified the sequence of amino acids on β4 integrin that interacts with 12-LOX.
- Preliminary experiments conducted to show the feasibility of disruption of the interaction of 12-LOX with β4 integrin in PC-3 cells with ectopic expression of the cytoplasmic tail sequence of β4 integrin. This resulted in the reduction of the biosynthesis of 12-HETE as well as tumor growth from PC-3 cells in experimental animals.

Reportable Outcomes:
NONE

Conclusions:
Interaction of 12-LOX with the cytoplasmic tail of β4 integrin results in the disruption of hemidesmosomes. Our data presented here demonstrates the potential to exploit the interaction between 12-LOX and β4 integrin using ectopically expressed cytoplasmic tail sequence of the integrin to modulate tumor growth.

References:
1. Nievers MG, Schaapveld RQ, Sonnenberg A. Biology and function of hemidesmosomes. Matrix Biol 1999;18(1):5-17.
2. Jones JC, Hopkinson SB, Goldfinger LE. Structure and assembly of hemidesmosomes. Bioessays 1998;20(6):488-94.
3. Tang K, Finley RL, Nie D, Honn KV. Identification of 12-lipoxygenase interaction with cellular proteins by yeast two-hybrid screening. Biochemistry 2000;39(12):3185-91.
4. Tang K, Nie D, Cai Y, Honn KV. The β4 integrin subunit rescues A431 cells from apoptosis through a PI3K/Akt kinase signaling pathway. BiochemBiophysResCommun 1999;264(1):127-32.

Appendices:
NONE